

A novel method - amenable for high-throughput screening purposes - to quantify antiviral activity against viruses that induce limited CPE.

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Abstract

For antiviral screenings purposes, infection of cell cultures with the virus under study, should ideally result in the induction, within just a few days, of (nearly) complete CPE and allow the calculation of acceptable Z' factors ($> 0,5$). The human Corona virus (HCoV-NL63) causes only limited CPE on different cell lines (Schildgen *et al.*, J Virol Methods. 2006). Following infection of Vero118 cells, virus-induced CPE was too low to allow readout based on classical colorimetric methods (such as the MTS assay), even following prolonged incubation times (> 7 days). To develop an antiviral screenings-assay against NL63, we explored whether a death-cell protease substrate could be used instead. The substrate used is a quenched peptide (bis-AAF-R110) that releases a fluorophore upon proteolytic-cleavage by proteases; the latter released from death cells. Following different rounds of optimization a screening protocol was developed: Vero118 cells in 96-well plate format were infected with NL63 (MOI=0,01; 200 μ L cell culture; $2 \cdot 10^4$ cells/mL, IMDM 5% FBS medium). Cultures were subsequently incubated for 5 days at 35°C after which 20 μ L of the peptide solution was added. Fluorescence was quantified 2 hr after incubation at 37°C. A roughly 3-fold increase in fluorescence intensity in the infected cultures was observed as compared to the uninfected cultures with a low well-to-well variability. Z' factors calculated from different experiments were in the range of 0.6 – 0.8, indicating excellent assay quality. An anti-ACE-II polyclonal antiserum (that prevents coronavirus infection in cell cultures) was used as a positive control and allowed to validate the assay for antiviral screening purposes. In conclusion, in conditions where a viability staining is inadequate to quantify virus-induced CPE, a novel simple and convenient method that detects cell-death and that is suitable for high-throughput screening purposes can be employed.

Introduction

Viral infections remain a major burden for human health world-wide. While it is difficult to estimate the total number of disease causing viral infections, the mortality caused by some specific viral pathogens is well described. Today, antiviral drugs are only available for the treatment of infections with (i) HIV, (ii) some DNA viruses (herpes viruses, hepatitis B virus, poxviruses) and (iii) a small number of RNA viruses (HCV, RSV, and influenza virus). Decades of rigorous effort were needed to develop these drugs and specific therapy is still lacking for the treatment of infections by all other RNA viruses, leaving supportive care as the only option.

A major strategy to identify novel antivirals is to screen compound libraries using phenotypic assays. As in other disease areas the contribution of phenotypic screening to the discovery of first-in-class small-molecule drugs exceeds that of target-based approaches (Swinney and Anthony, 2011). The main phenotype used in antiviral assays is the development of a virus induced cythopathic effect (CPE). If a virus infection renders sufficient CPE in cell culture standard viability assays (ex. MTS reduction) screens are feasible to detect viral and antiviral activity. However if the CPE is limited than more complicated and expensive tools are needed (ex ATP quantification).

Previously a method was reported to quantify cell toxicity. This method uses a “dead cell protease substrate”, which contains a fluorophore (R110) quenched by the covalent linkage of two Ala-Ala-Phe peptides (bis-AAF-R110). The method can be used to detect cell death since proteolytic-cleavage by proteases, released from dead cells, restores the fluorophore (Niles *et al.*, 2007) (Figure 1). Here we developed this method further for the quantification of limited CPE. The read-out is homogeneous and rather inexpensive and therefore amendable to medium- or high- throughput screening campaigns. Here we describe the method as a solution to screen for inhibitors of human coronavirus. We employed the human Coronavirus NL63 as a model system since it uses the same cellular receptor as the SARS-virus (ACE2). NL63 causes only limited CPE on different cell lines (Schildgen *et al.*, 2006) making CPE quantification troublesome with classical viability staining (ex MTS reduction).

Materials and Methods

The bis-AAF-R110 peptide was purchased from Eurogentec (Luik, Belgium). Both the human coronavirus NL63 strain and the Vero118 cell line were kindly provided by Ron A. Fouchier (Erasmus Medical Center, Rotterdam, The Netherlands). The NL63 strain was isolated from an 8-mo-old boy suffering from pneumonia (Fouchier *et al.*, 2004). Vero-118 cells are a subclone of Vero-WHO cells (Kuiken *et al.*, 2004), and are cultured in Iscove's Modified Dulbecco's Medium (IMDM Invitrogen cat no 21980-032) with 10% FBS, 100 IU penicillin/mL and 100 µg streptomycin/mL. Cells are split ¼ twice weekly. Anti-ACE-2 IgG was obtained from R&D systems (cat no AF933), solubilized in DPBS at 0.2 mg/mL and stored at -20°C until use.

After different rounds of optimization the following antiviral protocol was developed: Vero-118 cells in 96-well plate format are infected with NL63 (MOI=0.01, 200 µL cell culture, 20,000 cells/well, IMDM 5% FBS medium). Cultures are subsequently incubated for 5 days at 35°C after which 20 µL of

the peptide solution (16 μ M final concentration) is added. Fluorescence is measured after 2 hr incubation at 37°C using parameters for rhodamine fluorescence read-out.

For the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt) reduction assay an MTS/Phenazine methosulphate (PMS) stock solution (2 mg/mL MTS (Promega) and 46 μ g/mL PMS (Sigma) in PBS at pH 6-6.5) was diluted 1/20 in MEM (Invitrogen cat no 21090-022). Medium was aspirated from wells and 70 μ L of MTS/PMS solution was added. After 1-2 h incubation at 37°C absorbance was measured at 498 nm.

Results

In a first experiment a virus titration of two different virusstocks of NL63 was carried out and on day 6 p.i. read-out was performed by (i) microscopic scoring of the CPE and (ii) by using the CytoTox-Fluor™ Cytotoxicity Assay (Promega cat no G9260). This CytoTox-Fluor assay has been developed to quantify cell-death based on the cleavage of the bis-AAF-R110 peptide by proteases released from death cells (Niles et al., 2007). In this experiment the assay was used according to the manufacturers' instructions. Results are presented in Figure 2. For both virusstocks a very good correlation between the observed levels of CPE and the increased levels of fluorescence are observed. This indicates that the release of proteases by cultures that develop CPE and the subsequent cleavage of the bis-AAF-R110 peptide to its fluorescent substrate can be used to quantify CPE. When a similar experiment was carried out using other methods to detect cell viability such as the MTS reduction method (Figure 3) or the quantification of LDH release (data not shown), no significant difference between infected and non-infected cell cultures could be observed.

Next experiments were designed to optimize the assay for screening purposes. The optimal condition obtained was: 20,000 cells/well of Vero-118 cells infected with NL63 (MOI = 0.01 CCID₅₀/cell) in IMDM medium supplemented with 5% FBS medium. Upon infection cultures are maintained for 5 days at 35°C after which 20 μ L of the peptide solution (16 μ M final concentration) is added. Fluorescence is measured after 2 hr incubation at 37°C using parameters for rhodamine fluorescence read-out. The amount of peptide used under these conditions is 3x lower than proposed by the manufacturer of the Cytotox-Fluor assay. Using lower levels of peptide results in a decreased signal but the signal-to-noise ratio remains sufficient high to be used under screening conditions (see infra). The main reason to lower the amount of peptide is to make a screening campaign affordable.

A batch of bis-AAF-R110 peptide was next custom prepared. The peptide (MW 910 g/mol) was obtained as a light yellow powder, with > 95% purity as determined by HPLC and was stored at -20°C until use. Peptide stock solutions were prepared in DMSO at 100 mM and again stored at -20°C until use.

In a subsequent experiment we compared the performance of the custom synthesized peptide stock with that of the CytoTox-Fluor kit. In addition we evaluated the antiviral activity of a known SARS inhibitor i.e. Anti-ACE-2 IgG. This antibody inhibits SARS virus replication by binding ACE-2 and preventing the virus to enter the cell (Li et al., 2003; Ivens et al., 2005). The experiment was set-up as a standard antiviral experiment in which the antibody was diluted in medium at different concentrations in duplicate after which virus and cells were added. Untreated infected (virus controls), and treated uninfected (cell controls) cultures were included on each plate (6 wells each). On day 5 p.i. one plate was analyzed using the CytoTox-FLUOR kit according to manufacturers'

protocol (except that the peptide was used at a 3x lower concentration). In brief the peptide solution was diluted 1/600 in assay buffer (as supplied with the kit) and added to the 96 well plate at 20 μ L/well. The other plate was analyzed in parallel using the custom made peptide. To this end a 100 mM DMSO stock of the peptide was diluted 1/600 in assay medium and 20 μ L/well was added (final peptide concentration 16 μ M). Plates were incubated at 37°C for 90' and following thorough shaking the plates were read. Results are presented in **Error! Reference source not found.**. The performance of the custom prepared peptide batch was comparable to that of the CytoTox-FLUOR kit. A Z' factor of 0.75 and 0.85 was calculated in this experiment for the custom prepared and commercial peptide respectively. A Z' > 0.5 indicates sufficient quality for screening (Zhang *et al.*, 1999). The assay was validated for antiviral evaluation purposes using Anti-ACE-2 IgG and control IgG. Our results show that this antibody inhibits *in vitro* NL63 replication with an EC₅₀ ~0.3 μ g/mL (**Error! Reference source not found.**). The Anti-ACE-2 IgG was highly selective as no adverse effect on the cell layers could be observed microscopically (data not shown). The antiviral activity of Anti-ACE-2 IgG observed here was ~3x more pronounced than the activity against the SARS coronavirus (Li *et al.*, 2003; Ivens *et al.*, 2005). This is not unexpected since both viruses are known to bind the ACE2 receptor differently and have evolved independently in their ability to use it for cellular entry (Wu *et al.*, 2011).

Finally two independent antiviral assays were carried out using the custom prepared peptide. In each assay four 96 well plates were used with 6 infected and 6 uninfected wells in each plate. Fluorescence was quantified on day 5 p.i. and resulted in a Z'-factor of 0.63 (Figure 5).

Discussion

We demonstrate that a method, previously validated for quantifying cell toxicity (Niles *et al.*, 2007), can also be used to quantify virus induced CPE and antiviral activity under conditions suitable for screening. The assay principle relies on the detection of proteases released from death cells by using the bis-AAF-R110 peptide, which upon cleavage, turns fluorescent. The main advantage of this method, in comparison to cell viability measurements (such as the MTS reduction or LDH release methods), is that it allows to readily detect limited virus induced CPE. This allows using the method for antiviral screening campaigns with viruses that cause only limited levels of CPE. In addition the assay is highly homogenous (no washing steps) and affordable. In our hands the cost of substrate is ~€0.03/well. An additional advantage is the non-destructive nature of the method, allowing microscopic inspection and simultaneous or sequential use of other assay methods in the same well (for example to evaluate toxic or anti-proliferative effects of the compounds).

Acknowledgments

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Figure Legends

Figure 1: A novel assay principle to quantify virus induced CPE

Figure 2: Two different virusstocks (A and B) were titrated in a 96 well plate using the standard protocol. At day 6 p.i. titrations were read by microscopic scoring and with the CytoTox-Fluor™ Cytotoxicity Assay (Promega cat no G9260). CPE scoring was done on a scale from 0-1 where 0 indicates no CPE and 1 indicates CPE. Each measurement was performed in duplicate.

Figure 3: Comparing MTS Reduction with bis-AAF-R110 cleavage. A standard antiviral assay was carried out as described and 5 days p.i. the CPE was quantitated using either MTS reduction (Left) or bis-AAF-R110 cleavage (CytoTox-Fluor™ assay) (Right). Only the bis-AAF-R110 cleavage assay allows quantification of the limited amount of CPE.

Figure 4: Assaying Z' factors in virus infected Vero118 cells using either (A) the Cytotox-Fluor™ kit or (C) a custom-prepared bis-AAF-R110 peptide. Data are means and +- SD for 6 wells / condition. Panels B and D demonstrate the effect of Anti-ACE-2 IgG on the replication of NL63 using the Cytotox-Fluor™ kit or the custom-prepared bis-AAF-R110 peptide respectively.

Figure 5: Assay quality under HTS conditions using the custom prepared bis-AAF-R110 peptide. Two independent antiviral experiments were performed each using four 96 well plates. The individual results of the infected and uninfected control wells (total number of wells/condition is 48) as well as the average and Z' are presented.

Figures

Figure 1

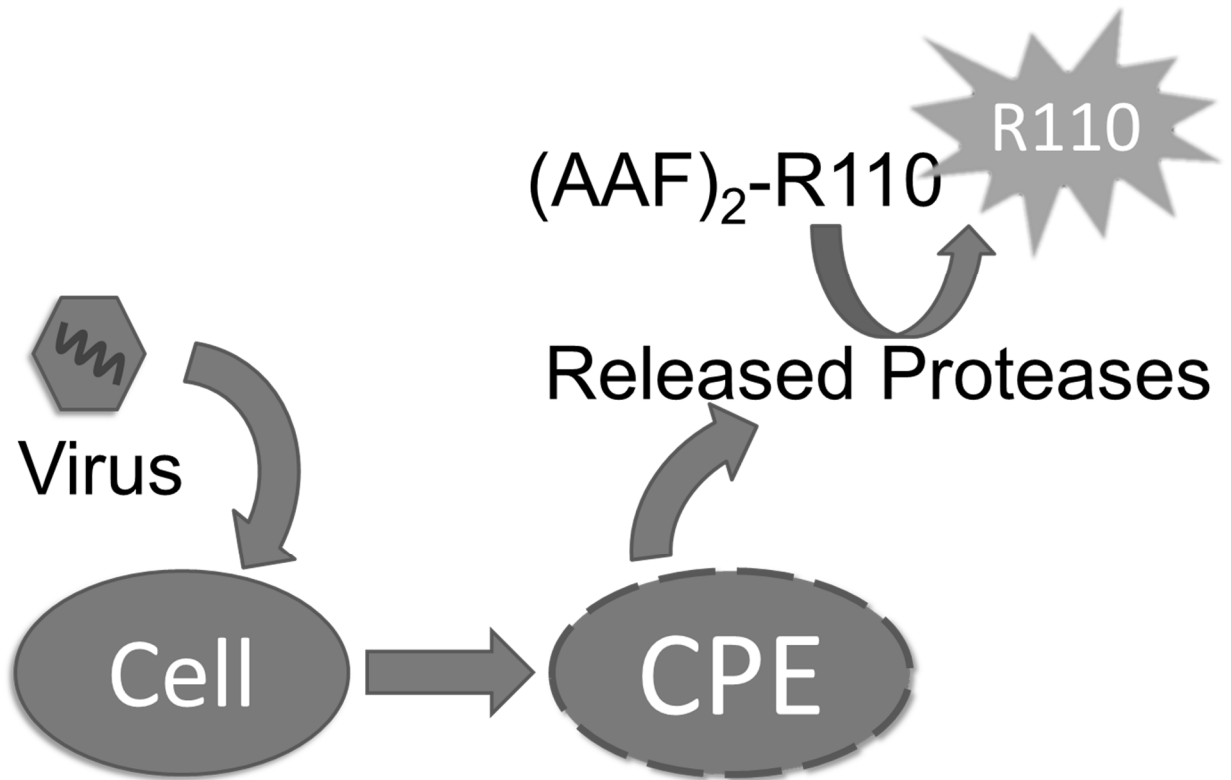


Figure 2

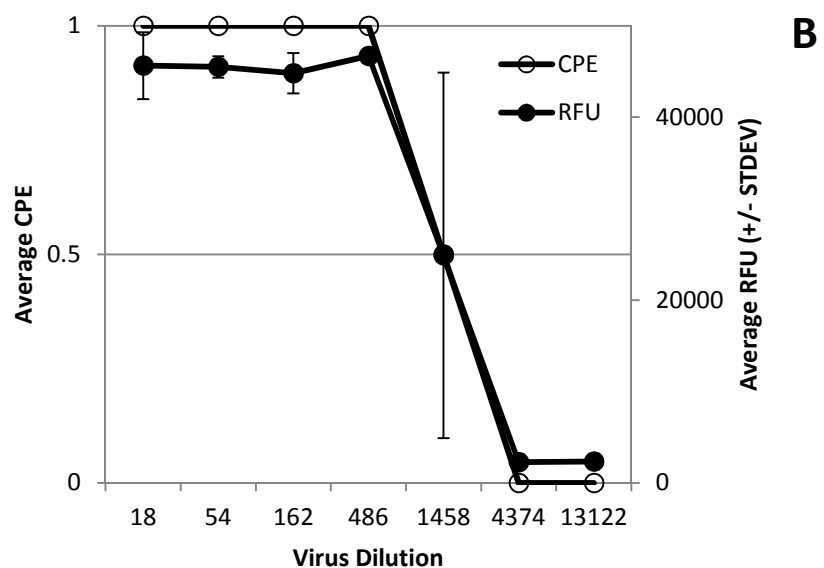
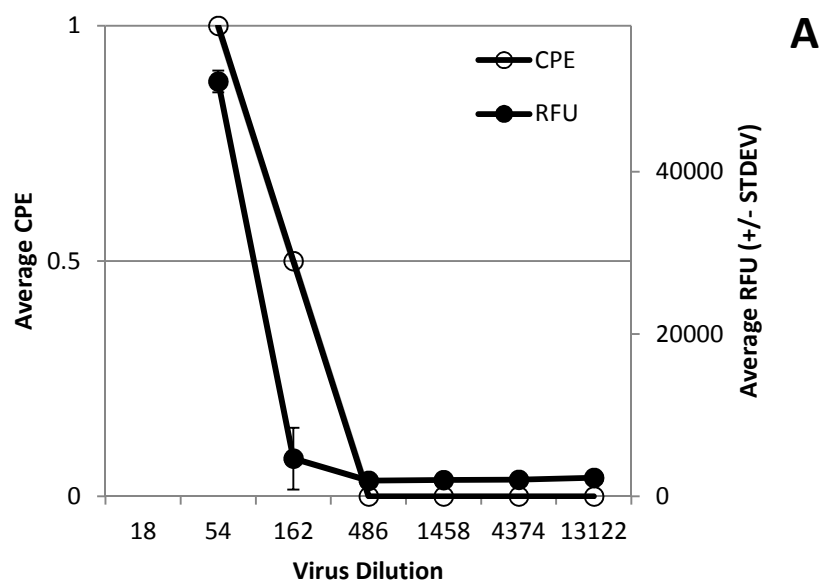


Figure 3

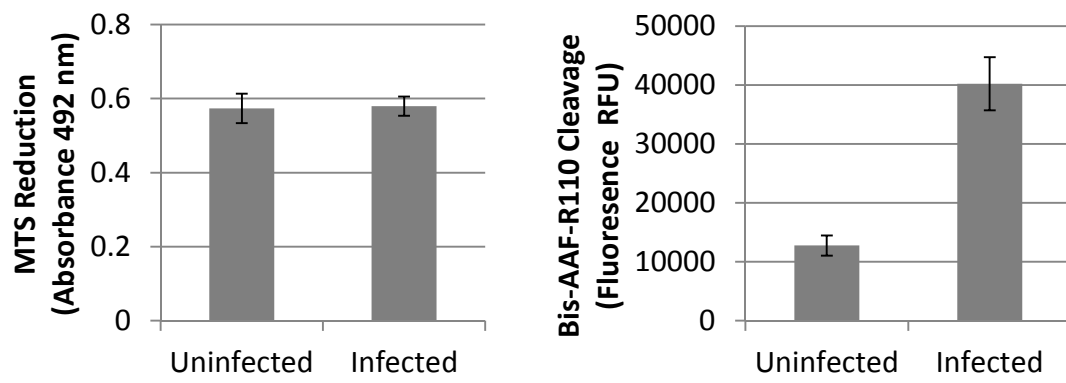


Figure 4

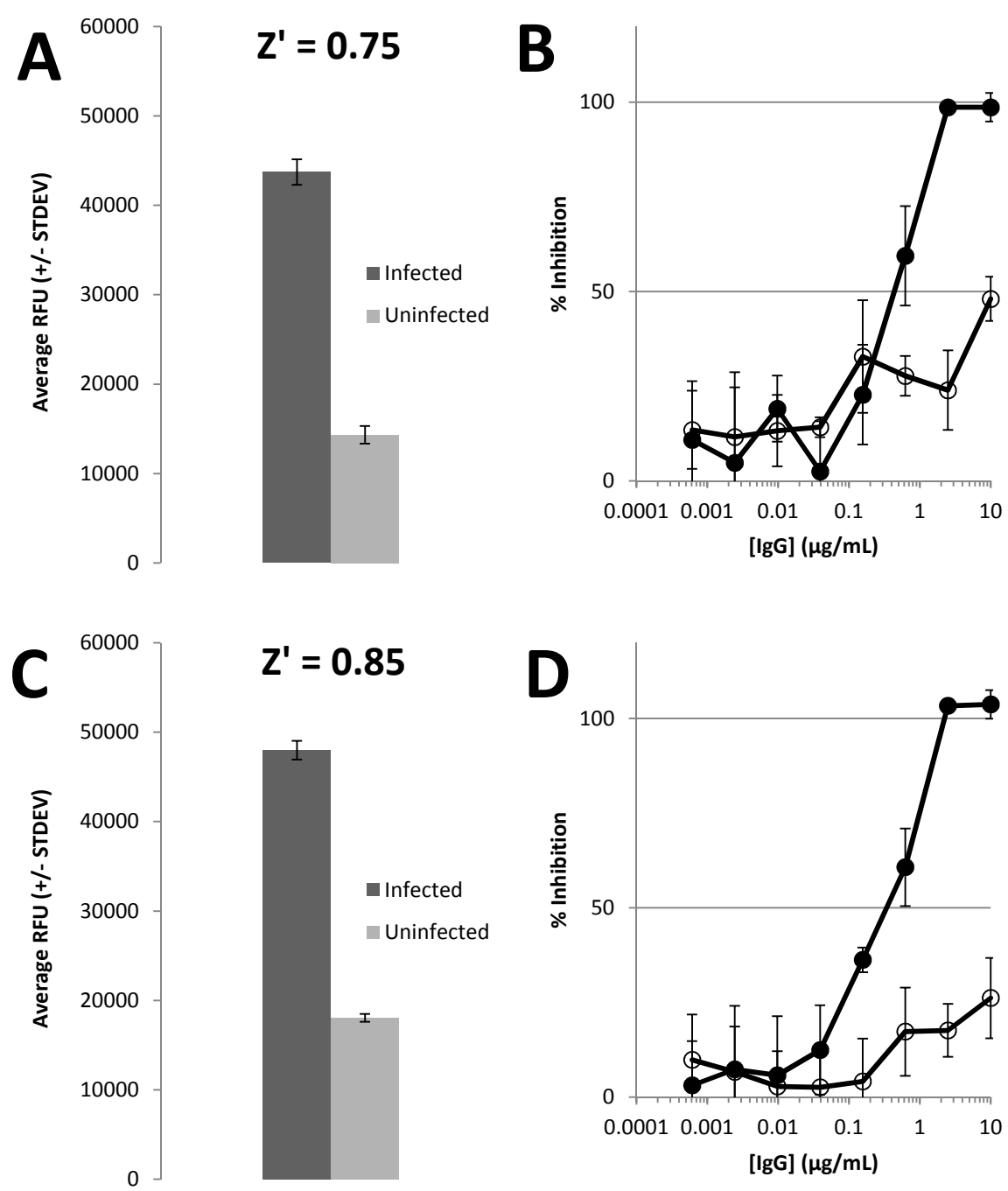


Figure 5

